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The subject of this grant was the development of a novel class of anticancer agents that induces the degradation of specific proteins by causing them to bind in a stable complex with the chaperone molecule hsp90. This was to be accomplished by synthesizing hybrid drugs comprised of the hsp90-binding drug geldanamycin covalently joined to a high affinity ligand for the protein to be degraded. The goal of this grant was to pilot this idea by making geldanamycin-estrogen hybrids to target the estrogen receptor. We succeeded in synthesizing several families of such hybrids, in determining characteristics of the linker moieties required for activity and in identifying compounds that selectively inhibit estrogen receptor, androgen receptor and various members of the PI3 kinase family. Furthermore, we have shown that a compound that selectively degrades estrogen receptor has selective cytotoxic activity against ER-containing MCF-7 breast cancer cells and that a corresponding compound with activity against androgen receptor selectively inhibits the growth of prostate cancer cells.

We are currently involved in the preclinical development of these compounds and hope to collaborate with the NCI and the pharmaceutical industry in bringing the most promising compounds to phase I clinical trial.

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INTRODUCTION

The subject of this grant was the development of a novel class of anticancer agents that induces the degradation of specific proteins by causing them to bind in a stable complex with the chaperone molecule hsp90. This was to be accomplished by synthesizing hybrid drugs comprised of the hsp90-binding drug geldanamycin covalently joined to a high affinity ligand for the protein to be degraded. The goal of this grant was to pilot this idea by making geldanamycin-estrogen hybrids to target the estrogen receptor. We succeeded in synthesizing several families of such hybrids, in determining characteristics of the linker moieties required for activity and in identifying compounds that selectively inhibit estrogen receptor, androgen receptor and various members of the PI3 kinase family. Furthermore, we have shown that a compound that selectively degrades estrogen receptor has selective cytotoxic activity against ER-containing MCF-7 breast cancer cells and that a corresponding compound with activity against androgen receptor selectively inhibits the growth of prostate cancer cells.

We are currently involved in the preclinical development of these compounds and hope to collaborate with the NCI and the pharmaceutical industry in bringing the most promising compounds to phase I clinical trial.

BODY

Two classes of natural products, the ansamycins (herbimycin A (HA) and geldanamycin (GM), and radicicols, represent novel drugs that bind to members of the Hsp90 family and inhibit protein refolding. They bind to a deep, highly conserved pocket in the aminoterminal portion of these chaperone proteins. This pocket is homologous to the helicase ATP-bind site and binds ADP and ATP with low affinity. Binding of ATP to this pocket is required for the final step in chaperone-mediated refolding and release of Hsp90 from the refolded protein. ADP binding prevents this process. The result of ansamycin binding is the selective degradation of a small subset of proteins that are dependent of Hsp90. These include transmembrane tyrosine kinases, Raf, and steroid receptors. Degradation is ubiquitin- and proteasome dependent. The most sensitive targets that we and others have identified are the HER-family of tyrosine kinases and the met tyrosine kinase.

The issues we are currently addressing include characterization of the cellular effects of these drugs, determination of whether regulation of hsp90 function plays a role in physiologic regulation of signal transduction and whether ansamycins can be exploited clinically. This grant dealt with the last issue.

We considered whether ansamycins could be used as anticancer drugs. They are potent inhibitors of cancer cell growth, but their mechanism of action suggests that they would be quite toxic. We conceived of three possibilities for their use in the clinic:

1. Treatment of tumors that depend on a target that is very sensitive to GM. These would include tumors in which the HER2 gene is amplified and those containing HER2\HER3,4 heterodimers. We have recently shown that a GM derivative, 17-allylaminogeldanamycin, is

very active in breast cancer xenografts that overexpress HER2. This drug is currently being tested in Phase 1 clinical trial.

2. Use in combination with radiation or cytotoxic chemotherapy. Inhibition of protein refolding by these drugs suggests that they could be used as radiation of chemotherapy sensitizers. Animal and cell culture models are being used to test this possibility. We have recently shown that ansamycins inhibit Akt activation and that this inhibition sensitizers HER2-driven breast cancers to taxanes and anthracyclines in tissue culture and in murine xenograft models. These data are being used to plan phase 1 and 2 clinical trials of geldanamycin derivatives in combination with chemotherapy.

3. The third possibility was the subject of this grant.: (Figures illustrating these findings are indexed to the publications in the appendix and are discussed in the Research Accomplishments section.)

In collaboration with Dr. Samuel Danishefsky, we proposed that derivatives of ansamycins that induce the selective degradation of specific proteins can be synthesized. These molecules would consist of ligands that bind with high affinity to targeted proteins linked covalently to an ansamycin. The hybrid drug would induce formation of a stable complex comprised of hsp90, drug and target protein. This complex would be inactive enzymatically (in the case of steroid receptors) and a target for degradation in the proteasome.

There are two levels to this strategy. First, proteins that are sensitive to the parent compound can be targeted. The goal is to create an agent that has selective potency against that protein compared to other targets of GM. Such an agent would presumably be less toxic than GM. Thus far, we have synthesized and characterized three classes of drug with relative selectivity targeted to HER-kinases, estrogen receptor and androgen receptor, respectively.(See below.) Second, proteins that are insensitive to the parent compound can be targeted. The hope is that any heterodimer of hsp90 and a particular protein will be inactivated enzymatically and, perhaps, subject to degradation. We have synthesized GM-hybrids targeted to PI3 kinase and to various immunophilins. We have succeeded in identifying GM-PI3kinase inhibitor hybrids with selective activity against different members of the PI3 kinase family. (3).

Another strategy is based on the fact that transmembrane tyrosine kinases heterodimerize when activated. We synthesized GM dimers joined at the 17-position by linkers of various lengths. The ideas is that some selectivity might be achieved as a function of type and length of linker. This strategy has proven to be successful. (4)

Statement of Work

Technical Objectives

1. Synthesis of ansamycin derivatives coupled to targeting molecules.
2. Test whether the molecules synthesized in Aim 1 direct the specific degradation or inhibition of the targeted protein.
3. Test the biologic effects of the hybrid drugs.

We successfully synthesized several classes of hybrid drugs in which geldanamycin was covalently attached to a ligand that binds to a particular targeted protein (1-3). The idea underlying this work is that such hybrid compounds will cause the formation in the cell of heterodimers of the targeted protein and Hsp90. Our previous work suggested that such dimers might be selectively degraded in the proteasome. Subsequent work suggested that even if the targeted protein were not degraded, it would be sequestered in an abundant sink of cytosolic Hsp90 and thus be inactivated. This has proven to be the case for androgen-geldanamycin hybrids.

KEY RESEARCH ACCOMPLISHMENTS

(The papers that were generated by and that describe this work are listed below and also form the appendix attached to the grant. Figures referred to in the text are in the cited papers in the appendix.)

Molecules synthesized and characterized

1. Geldanamycin-estradiol hybrid drugs

A family of such drugs attached by linkers with different properties were synthesized and characterized (1) (Fig1-3, Kuduk, 1999). Activity is highest with unsaturated linkers 4-6 carbons long. (Table I, Kuduk, 1999) One of these has been selected as a lead compound. It is 10-20 fold less potent than geldanamycin against estrogen receptor, but 200-1000 fold less potent against androgen receptor, IGF-I kinase and Raf. It still induces HER kinase degradation with the same potency as displayed against estrogen receptor. (Fig. 2, Kuduk, 1999) Thus, this represents a partially selective compound. It may be useful for the treatment of breast cancer, as both HER2 and estrogen receptor are key targets in this disease. Preliminary testing of the drug shows that, although it is less active than GM, it is selectively toxic to breast cancer cells that contain estrogen receptor.. The drug is active against the estrogen receptor containing cell line MCF-7 but not against prostate cancer cell lines (Data not shown.). We regard this as proof of principle of original concept:that geldanamycin derivatives could be synthesized with different specificity than that of the parent molecule. Furthermore, we predict that the molecule we have synthesized will be less toxic than the parent and a potentially good drug for breast cancers expressing estrogen receptor, with or without HER2. We have now synthesized enough drug to test in animals. A maximally tolerated dose and effective of administration has been determined for the non-selective derivative, 17-allylaminogeldanamycin. We will use these findings to test the activity of estradiol-geldanamycin against MCF-7 xenografts.

2. Geldanamycin-testosterone hybrid drugs

Although not part of the original proposal, we have extended this work to synthesize an analogous family of testosterone-geldanamycin hybrids (2) (Fig. 2, Kuduk, 2000). These were much less potent than either GM or the estrogen-GM in inducing protein degradation (Table 1, Kuduk, 2000.). However, one testosterone-GM hybrid was a potent and selective inhibitor of prostate cancer cell growth. It inhibits the proliferation of tumor cells with wild type or mutated androgen receptor at doses much (30-100-fold) less than those required to inhibit cells without the receptor (Table 2, Kuduk, 2000). Furthermore, inhibition occurs in the absence of degradation of families of protein targets. Although the drug does not potently induce androgen receptor degradation, it causes a rapid loss of nuclear receptor and an accumulation of the receptor in the cytosol (Data not shown.). Our working hypothesis is that androgen receptor-Hsp90 dimers form and cannot translocate into the nucleus.

These data suggest that this drug will be a potent inhibitor of advanced prostate cancer with much less toxicity than the parent compound. We regard this compound as a very exciting discovery with potential clinical utility. We are now synthesizing a family of related derivatives. The most promising lead molecules will be made in large quantities and their activity will be assessed in animal models of androgen-dependent prostate cancer. We have shown that 17-allylaminogeldanamycin is quite active in one such model, CWR22 human prostate xenograft. The activity of androgen-receptor selective and unselective ansamycins will be tested in this model.

3. Geldanamycin-LY294002 Dimers

Phosphatidyl-3'-phosphokinase (PI3kinase) is a key molecule in the transduction of the growth factor signal. It does not bind to Hsp90 family members and is unaffected by ansamycins. In order to test whether we could target the inactivation of such proteins with ansamycin derivatives, we synthesized a family of molecules comprised of geldanamycin linked to a ligand for the p85 regulatory subunit of PI3kinase. (3) (Fig. 1, Scheme 1, Chiosis, 2001) We utilized LY294002, a drug (Eli Lilly) that binds to this subunit and inhibits the enzyme. We identified members of this family of molecules that inhibit PI3kinase activity in vitro in an Hsp90 dependent manner. Furthermore, these molecules show selectivity among PI3K family members. (Table 1, Chiosis, 2001.) Thus, we have identified selective PI3kinase and DNA-dependent kinase inhibitors.

These data support the idea that this technology can be used to create targeted inhibition of many proteins, including those that do not ordinarily interact with Hsp90. This work is preliminary. Current studies are focused on further characterizing members of this family of compounds and assessing whether they work in cells and how they affect tumor cell growth. Several members of the PI3k family, including ATM, ATR, DNA-PK, and PI3k play keys roles in regulating cellular proliferation and the response of the cell to DNA damage. Selective inhibitors could have multiple uses in the treatment of cancer and other diseases.

4. GM-dimers

During the course of this work, we developed another class of geldanamycin derivatives, not envisioned in the original application, that could be useful in the treatment of breast cancer. The HER2 transmembrane tyrosine kinase signals by homodimerizing or by forming heterodimers with the other members of the HER kinase family. These heterodimers are predicted to bind to two, apposed, Hsp90 molecules. We reasoned that geldanamycin dimers connected by linkers of specific lengths might selectively interact with particular activated HER kinase dimers.

A class of GM-dimers joined at the 17-carbon by linkers varying in length from three to twelve carbons were synthesized. (4) (Fig. 1 Zheng, 2000) The ability of these dimers to induce the degradation of a collection of GM-target proteins was evaluated. The dimers were less potent than GM or 17-allylaminoGM and potency declined with increasing linker length. (Table 1, Zheng, 2000.) The 4-carbon linked dimer (4C-GM) was selective for HER-family tyrosine kinases. It was much less active against Raf and steroid receptors and inactive against the IGF-I receptor (Fig 2A-C,.Zheng, 2000.) Selectivity was not secondary to the isolation of a generally weak agent which retained activity only against the most sensitive target(s). Selectivity was maintained when the drug was given frequently and at high concentration. (Fig. 2D, Zheng, 2000.) Furthermore, selectivity required intact dimer. Selectivity was lost when both or either of the ansa rings was opened (Table 1, Zheng, 2000.).

Identification of a HER kinase selective drug was of great interest, given the proven role of these enzymes in multiple tumors and the known sensitivity of breast cancers with HER2 gene amplification to an anti-HER2 antibody. Accordingly, we have tested the effects of 4C-GM on cancer cells. The drug is a potent inhibitor of the growth of breast cancer cell lines with HER kinase. The drug causes hypophosphorylation of RB, and G1 arrest. G1 arrest is followed by differentiation and then apoptosis. (Fig 3. Zheng et al and data not shown.) These effects are very similar to that of the parent ansamycin, except that growth inhibition is accompanied by loss in HER-kinase expression but not in loss of the expression of other GM targets such as Raf. As opposed to GM, 4C-GM causes a much more specific G1 block. Taken together, the data suggests that this drug is more selective than GM, potently inhibits tumor cell growth, and is likely to have fewer side effects. These and other data from our laboratory also suggest that the key effects of HER2 on G1 transit are not mediated via the Ras-Raf-MAP kinase pathway. Other data we have generated outside the scope of this grant indicates that the primary effects of HER2 on growth are mediated by activation of PI3kinase, akt kinase dependent pathways.

The properties of this drug and the importance of its target have led us to identify 4C-GM as a lead compound and to put high priority in its development. Current goals include studies on the biochemical mechanism of action, testing of the drug against breast cancers in HER2 transgenic mice and the synthesis and testing of other GM-dimers with different linkers.

REPORTABLE OUTCOMES

In other studies, not part of this grant, we have characterized some of the mechanisms whereby geldanamycin kills tumor cells. We have developed therapeutic models for the testing of ansamycins in xenograft and transgenic mouse models of breast cancer and will use the same models in the testing of selected, potent, ansamycin derivatives that we have generated and described in this report.

In collaboration with the NCI, we are conducting a phase I study of 17-allyaminogeldanamycin. We plan to initiate phase 2 trials in 2002. Some of the molecules generated by our work will be targeted for accelerated preclinical and clinical development. Our current studies with a non-selective parent compound will act as models in this effort.

Publications Generated By These Studies

Chiosis G, Rosen N and Sepp-Lorenzino L. Synthesis and Evaluation of Several LY294992-Geldanamycin Heterodimers as Selective Inhibitors of the PI3K and PI3K-related family. Bioorganic and Medicinal Chemistry Letters 11 (909-913) 2001.

Kuduk, SD, Harris, CR, Zheng FF, Sepp-Lorenzino L. Ouerfelli O., Rosen N, Danishefsky SJ. Synthesis and Evaluation of Geldanamycin-Testosterone Hybrids. Bioorganic and Medicinal Chemistry Letters 10 (1303-1306) 2000.

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CONCLUSIONS

In the course of our work, we have generated four classes of ansamycin derivatives that are selective for estrogen receptor, HER kinase, PI3kinase family members and androgen receptor. The first two will serve as lead molecules for the development of drugs useful in the treatment of breast cancer; the last will be developed for the treatment of prostate cancer. The PI3 kinase inhibitors may be useful in multiple diseases.

This work establishes the possibility of using this strategy to target the selective inactivation or degradation of particular proteins. Moreover, in other studies, in murine models, we have established that ansamycins cause degradation of target proteins and have antitumor activity at nontoxic doses. These studies have led to the clinical testing of the parent molecule and provide a model for the preclinical development of the second and third generation drugs described herein. The weight of the data suggest to us that compounds that bind the Hsp90 pocket may be useful anticancer agents.

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Bioorganic & Medicinal Chemistry Letters 11 (2001) 909–913

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LETTERS

LY294002-geldanamycin Heterodimers as Selective Inhibitors of the PI3K and PI3K-related Family

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Abstract—Several LY294002-GM heterodimers were synthesized with the intent of modulating their activity in the presence of hsp90 and thereby creating selective inhibitors of PI3K and PI3K-related family. © 2001 Elsevier Science Ltd. All rights reserved.

The specificity and affinity of a ligand–protein interaction can be modulated by borrowing additional surface contacts from another protein.[1] By linking a weak and/or unselective ligand to the ligand of a physiologically abundant protein one could envision increasing the efficacy of the interaction if the created contact among the protein partners is favorable. In this fashion, selective inhibition of proteins that have similar pockets could be achieved.

Phosphoinositol-3 kinase (PI3K) and a newly emerging subfamily called PI3K-related kinases share a similar carboxy-terminus catalytic domain.[2] Given the sequence homology in the kinase domain of these proteins it is expected that they will all be inactivated by the known PI3K inhibitors wortmannin and LY294002.[3] The PI3K kinases consist of enzymes composed of various catalytic subunits of the $p110\alpha$, $p110\beta$, and $p110\gamma$ and the yeast homologue Vps34p types. The $p110\alpha$ and $p110\beta$ subtypes form tight heterodimers with a p85 regulatory unit, the assembly being activated by binding to autophosphorylated growth factor receptors or their substrates. Other PI3Ks (e.g., $p110\gamma$) seem to propagate signals from seven transmembrane helix receptors as they are activated by G-protein $\beta\gamma$-subunits. The PI3K-related proteins, ataxia-telangiectasia mutated (ATM), ataxia-telangiectasia related (ATR) and DNA-dependent protein kinase (DNA-PK) play important roles in checkpoints that operate to permit cell survival following many forms of DNA damage. Emerging experimental evidence suggests that although these proteins posses similar substrate specificity, they exhibit overlapping but distinct functions in vivo. ATM, ATR, and DNA-PK have been involved in regulating p53, and additionally, ATM has been found to play an important role in the control of the non-receptor tyrosine kinase Abl. Their counterparts in yeast have been identified as rad3p, Mec1p, Tel1p, and Esr1p. Another PI3K-related protein that possesses the kinase domain is FRAP (mTOR). FRAP is homologous to the yeast Tor1p/2p believed to control aspects of several diverse cellular functions, protein stability, translocation and translation as well as glucose metabolism. For example, FRAP controls pathways that are activated by transmembrane receptors, such as regulation of $p70^{s6}$ kinase.

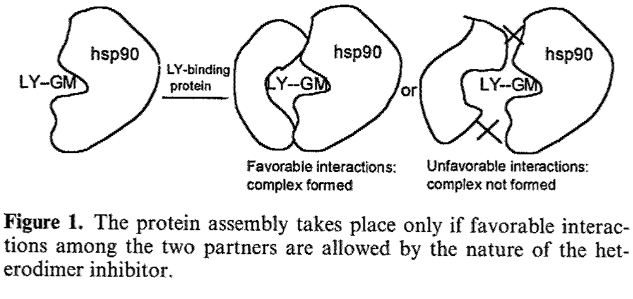
hsp90
LY–GM
LY-binding protein
hsp90
LY–GM
or
hsp90
LY–GM
Favorable interactions: complex formed
Unfavorable interactions: complex not formed

Figure 1. The protein assembly takes place only if favorable interactions among the two partners are allowed by the nature of the heterodimer inhibitor.

Selective inhibitors of the members of this family would constitute important tools for investigating the regulatory functions mediated by each protein. Additionally, due to the radiosensitive phenotypes exhibited by cells defective in ATM, ATR, or DNA-PK, inhibitors of these kinases might enhance the cytotoxic effects of ionizing radiation or DNA damaging cancer chemotherapeutic agents.

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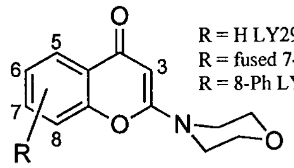
In this paper we describe a new strategy for the synthesis of selective PI3K inhibitors. An LY294002-like molecule was chosen as the kinase domain inhibitor and the hsp90 chaperone[4] as the interaction modulator protein (Fig. 1). The choice of this protein comes from its abundance in the cytosol and the existence of a high affinity binder, geldanamycin (GM).[5] Additionally, hsp90 is expressed at 2- to 10-fold higher levels in tumor cells compared to their normal counterparts. One would expect that given the abundance of hsp90 and the high affinity of GM for the protein, heterodimers of the LY294002-GM type would predominantly bind to hsp90 and be presented to the PI3K family only if the interactions in this tertiary complex are favorable (Fig. 1).

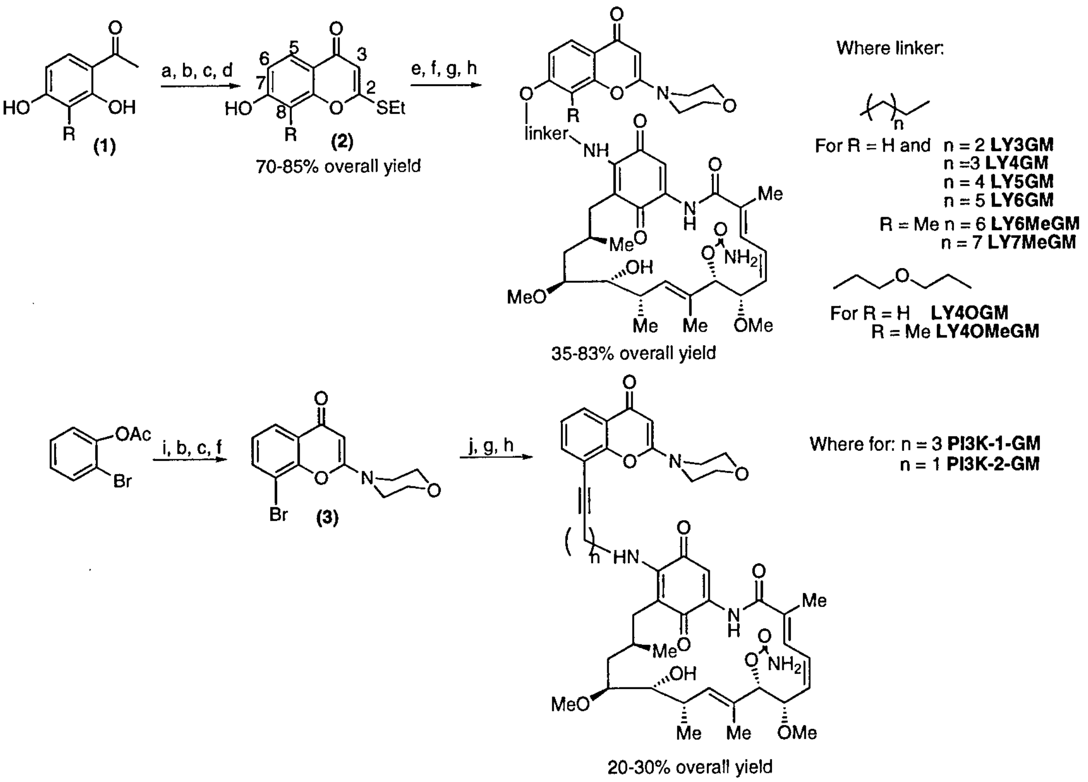
A very important factor in determining the nature of the interaction would be the length, nature, and mode of attachment of the linker. A preferential complex formation would result in selective binding and, therefore, compounds with more restricted cellular effects than the parent LYs.



R = H LY292223 IC$_{50}$ = 5 μM
R = fused 7-8 Ph, LY293646 IC$_{50}$ = 6 μM
R = 8-Ph LY294002 IC$_{50}$ = 1.4 μM

Due to the relatively similar inhibitory effects of the 7- and 8-substituted derivatives in the LY series, we chose LY292223 as the kinase domain-binding skeleton. OH or Br derivatized 2-hydroxy-3-phenylacetophenones were chosen to be the starting points for the facile reconstruction of such a skeleton (Scheme 1). These functionalities permit the attachment of several linkers through a Mitsunobu or a modified Sagatoshira reaction, respectively. Construction of the LY-skeleton was performed, with the appropriate modifications, based on the method published[6] by the Eli Lilly group. Condensation of the benzyl protected hydroxy-acetophenones (**1**) with carbon disulphide in the presence of potassium *t*-butoxide in benzene gave the corresponding thionocoumarins. These were converted to thioethers by alkylation with iodoethane and potassium carbonate.

If the linkers were to be attached through the Mitsunobu reaction, the benzyl group was first deprotected using boiling trifluoroacetic acid. Alkylation was carried out on the resulting phenols (**2**) with several *N*-Boc protected amino alcohols in a mixture of toluene/dichloromethane. After the attachment of the linker, the 2-sulphinyl moiety was replaced by morpholinyl through a nucleophilic displacement by simple refluxing in morpholine under acetic acid catalysis. Having the LY-skeleton and the linker assembled, GM was introduced through the ability of this molecule to undergo smooth Michael reactions with primary amines.



Scheme 1. Synthesis of the LY-GM heterodimers. (a) BnBr, K$_2$CO$_3$, DMF, 60 °C, 16 h; (b) 10 equiv *t*-BuOK, 3 equiv CS$_2$, benzene, 16 h; (c) EtI, K$_2$CO$_3$, acetone, reflux 30 min; (d) TFA, PhSMe, 70 °C, 16 h; (e) 3 equiv HO-linker-NHBoc, 1.3 equiv PPh$_3$, 1.3 equiv DEAD, toluene/DCM (10:1), 18 h; (f) morpholine, cat. AcOH, 75 °C, 2–3 h; (g) TFA/DCM (1:4), 1 h, rt; (h) GM, 3 equiv TEA, DMF, 15–24 h; (i) AlCl$_3$, 120 °C, 16 h; (j) 1.7 equiv *N*-Boc protected alkyl-ε-ynylamine, Pd(PPh$_3$)$_4$, PPh$_3$, CuI, morpholine.

If the attachment mode is the Sagatoshira reaction, the whole LY-skeleton is assembled first to give 2-morpholinyl-8-bromochromone (**3**), followed by addition of the *N*-Boc protected alkyl-ε-ynylamine linker. After standard Boc deprotection, the resulting amine was coupled to GM, giving the derivatives PI3K-1 and PI3K-2.[7]

To assess the effect of the mode of attachment and size of the linker on the activity of these dimers, we tested them for their ability to inhibit PI3K and DNA-PK, and additionally, to compete with solid-phase immobilized GM for hsp90 binding.

Inhibition of PI3-kinase by heterodimers[8]

The PI3K enzyme was immunopurified from insulin stimulated MCF-7 breast cancer cell lines using protein A immobilized anti-p85 antibody. Its ability to catalyze the phosphorylation of phosphatidylinositol by ATP in the presence and absence of drugs was determined. The hybrids LY3-GM, LY4-GM, LY5-GM, LY6-GM, and LY4O-GM showed significantly lower activity than the parent compound LY292223 (Table 1). Surprisingly, LY4OMe-GM, LY6Me-GM, and LY7Me-GM that differ from the above series just by the presence of a methyl group at the 8 position of the LY-skeleton, exhibited potencies comparable to the Lilly derivative, while the derivatives PI3K-1-GM, PI3K-2-GM, and LY6Me-GM were more potent. Free GM (at 500 μM) and DMSO (2.5% v/v) had no effect on the kinase activity of PI3K at the studied concentrations (data not shown).

Inhibition of DNA-PK by heterodimers[9]

A peptidic sequence derived from p53 was used as the substrate to determine the kinase activity of DNA-PK in the presence of the heterodimers. There is a consistent correlation between linker attachment site and potency of inhibition of DNA-PK and PI3-K. All the LYn-GM members are very weak inhibitors of PI3K with IC_{50}s over 300 μM, however, they inhibit DNA-PK at concentrations comparable to the parent Lilly compound. The most selective candidate, LY6-GM is over two orders of magnitude more active against DNA-PK than PI3K. The LYnMe-GM series shows only 1.5- to 16-fold selectivity, while, most surprisingly, the PI3K-n-GM series has the exact potency in inhibiting both proteins. GM (at 40 μM) and DMSO (8% v/v) had no effect on the activity of DNA-PK.

Table 1. Inhibition of PI3K activity and competition for hsp90 binding by the synthesized heterodimers

Compounds	IC_{50}, μM[a] for inhibition of PI3K activity	IC_{50}, μM[a] for inhibition of DNA-PK activity		EC_{50}, μM drug necessary to compete for 50% of Hsp90 a binding
		−hsp90 α	+ hsp90 α	
17AAG	>500	>40		1
WTM	ND	0.02		$\gg$50
LY292223	14 (±2)	4.3 (±0.6)		$\gg$50
LY3-GM	>500	28 (±1)		26
LY4-GM	200 (±12)	13.9 (±0.8)		10
LY5-GM	310 (±23)	10 (±0.4)	5 (±0.1)	2.5
LY6-GM	>500	6.1 (±0.2)		4
LY4O-GM	85 (±15)	33 (±1.8)		16
LY4OMe-GM	70 (±13)	12.2 (±1.8)		>50
LY6Me-GM	5 (±1.5)	3.7 (±1)		46
LY7Me-GM	28 (±3)	1.75 (±0.15)		28
PI3K-1-GM	13.5 (±2.5)	13.4 (±1.3)		$\gg$50
PI3K-2-GM	3 (±1)	3 (±0.2)		7

[a]Values are means of two experiments, standard deviation is given in parentheses. 17AAG is a GM derivative with similar potency.

Hsp90 binding assays[10]

GM immobilized on solid phase was utilized to determine the ability of the synthesized heterodimers to bind to the hsp90 α protein. Drugs were pre-incubated with protein and then added to the GM-beads. After an incubation time, the amount of protein left for binding to the solid support was separated and quantified. The ability of drugs to compete with the immobilized GM for 50% of the hsp90 was measured (Table 1). It was expected that the addition of a floppy linker on the GM molecule would diminish somewhat its affinity for hsp90. It is rather unexpected however, that some small variations in the linker substantially affected hsp90 binding. The only difference between LY5-GM and LY4O-GM is the substitution in the linker of one C atom with O, however, the latter is 6 times less active.

Modulation of LY5-GM binding to DNA-PK by hsp90

LY5-GM is the tightest hsp90 α binder and a modest DNA-PK inhibitor. It was, therefore, an attractive candidate to study the influence of hsp90 on kinase activity. Pre-incubation of the heterodimer with hsp90 α increased its inhibition of DNA-PK by 50% (Table 1). No effect on enzyme activity was observed in the samples that hsp90 was pre-incubated with DMSO (not shown).

The study points out how unpredictable and important a small modification in the nature, length, and mode of attachment of the linker can be. The presence of a methyl group at the 8 position of LY292223 seems to have a crucial role in determining the selectivity of the inhibitors. The methylated derivatives are good PI3K and DNA-PK inhibitors and have modest affinity for Hsp90. Meanwhile, compounds without the 8-methyl are good DNA-PK inactivators and maintain good hsp90 binding, however, PI3K is 3 log more resistant to their action. DNA-PK may be important for suppression of apoptosis. Therefore, compounds that selectively affect DNA-PK and not PI3K may be of therapeutic use in cancer and other disorders.

It is noteworthy that PI3K-2-GM has increased PI3-kinase and DNA-PK inhibitory effect compared to the parent Lilly derivative and binding for Hsp90 analogous to 17AAG.

Our study suggests that it is possible to modulate the activity of dual ligands, at least in in vitro systems. Additionally, it implies that the strategy outlined here can be used to generate a family of inhibitors that differentially inhibit members of the PI3K-family. Screenings in mammalian cell culture systems dependent on various PI3K-related members or in functional yeast hybrid systems will assess the in vivo activity of the heterodimers.

Acknowledgements

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7. LY6-GM: ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.18 (s, 1H), 8.03 (d, 1H, *J* = 8.8 Hz), 7.27 (s, 1H), 6.95 (d, 1H, *J* = 11.2 Hz), 6.88 (dd, 1H, *J* = 8.8 Hz, *J* = 2.2 Hz), 6.73 (d, 1H, *J* = 2.2 Hz), 6.58 (t, 1H, *J* = 11.4 Hz), 6.28 (bt, 1H), 5.84 (m, 2H), 5.42 (s, 1H), 5.19 (s, 1H), 4.76 (bs, 2H), 4.28 (m, 2H), 4.03 (t, 2H, *J* = 5.2 Hz), 3.81 (m, 4H), 3.56 (m, 2H), 3.45 (m, 6H), 3.35 (s, 3H), 3.29 (s, 3H), 2.71 (m, 2H), 2.37 (m, 1H), 2.05 (s, 3H), 1.77 (m, 10H), 1.48 (m, 4H), 1.25 (m, 2H), 1.00 (d, 3H, *J* = 6.9 Hz), 0.96 (d, 3H, *J* = 6.5 Hz). MS *m*/*z* 897.6 (M + Na).

LY5-GM: ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.16 (s, 1H), 8.03 (d, 1H, *J* = 8.8 Hz), 7.26 (s, 1H), 6.94 (d, 1H, *J* = 11.7 Hz), 6.88 (dd, 1H, *J* = 8.8 Hz, *J* = 2.2 Hz), 6.71 (d, 1H, *J* = 2.2 Hz), 6.57 (t, 1H, *J* = 11.4 Hz), 6.28 (bt, 1H), 5.86 (m, 2H), 5.47 (s, 1H), 5.18 (s, 1H), 4.82 (bs, 2H), 4.29 (d, 1H, *J* = 9.9 Hz), 4.03 (t, 2H, *J* = 6.0 Hz), 3.81 (m, 4H), 3.56 (m, 2H), 3.50 (m, 5H), 3.34 (s, 3H), 3.25 (s, 3H), 2.68 (m, 2H), 2.37 (m, 1H), 2.02 (s, 3H), 1.77 (m, 2H), 1.76 (s, 3H), 1.68 (m, 6H), 1.25 (m, 2H), 1.00 (d, 3H, *J* = 6.9 Hz), 0.96 (d, 3H, *J* = 6.5 Hz). MS *m*/*z* 883.8 (M + Na).

LY4-GM: ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.15 (s, 1H), 8.05 (d, 1H, *J* = 8.8 Hz), 7.27 (s, 1H), 6.94 (d, 1H, *J* = 12.0 Hz), 6.89 (dd, 1H, *J* = 8.8 Hz, *J* = 2.2 Hz), 6.73 (d, 1H, *J* = 2.2 Hz), 6.57 (t, 1H, *J* = 11.3 Hz), 6.32 (bt, 1H), 5.86 (m, 2H), 5.42 (s, 1H), 5.19 (s, 1H), 4.76 (bs, 2H), 4.30 (d, 1H, *J* = 9.9 Hz), 4.24 (bs, 1H), 4.06 (t, 2H, *J* = 5.0 Hz), 3.80 (m, 4H), 3.53 (m, 3H), 3.48 (m, 5H), 3.31 (s, 3H), 3.26 (s, 3H), 3.08 (m, 2H), 2.67 (m, 2H), 2.39 (m, 1H), 2.02 (s, 3H), 1.99 (m, 4H), 1.78 (s, 3H), 1.68 (m, 4H), 1.25 (m, 2H), 0.99 (d, 3H, *J* = 6.9 Hz), 0.94 (d, 3H, *J* = 6.5 Hz). MS *m*/*z* 869.8 (M + Na).

LY3-GM: ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.15 (s, 1H), 8.05 (d, 1H, *J* = 8.8 Hz), 7.27 (s, 1H), 6.92 (m, 2H), 6.82 (d, 1H, *J* = 2.2 Hz), 6.68 (bt, 1H), 6.56 (t, 1H, *J* = 11.3 Hz), 5.87 (m, 2H), 5.69 (s, 1H), 5.17 (s, 1H), 4.84 (bs, 2H), 4.29 (d, 1H, *J* = 9.9 Hz), 4.17 (t, 2H, *J* = 5.3 Hz), 3.82 (m, 5H), 3.69 (m, 1H), 3.53 (m, 5H), 3.42 (m, 1H), 3.34 (s, 3H), 3.25 (s, 3H), 2.68 (m, 2H), 2.42 (m, 1H), 2.21 (m, 2H), 2.00 (s, 3H), 1.71 (s, 3H), 1.69 (m, 3H), 1.25 (m, 2H), 0.97 (m, 6H). MS *m*/*z* 855.8 (M + Na).

LY4OMe-GM: ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.11 (s, 1H), 7.96 (d, 1H, = *J* = 8.7 Hz), 7.25 (s, 1H), 6.94 (d, 1H, *J* = 11.5 Hz), 6.88 (d, 1H, *J* = 8.7 Hz), 6.54 (m, 2H), 5.87 (m, 2H), 5.42 (s, 1H), 5.19 (s, 1H), 4.73 (bs, 2H), 4.27 (m, 3H), 3.93 (m, 2H), 3.86 (m, 6H), 3.71 (m, 2H), 3.49 (m, 6H), 3.34 (s, 3H), 3.26 (s, 3H), 2.68 (m, 2H), 2.32 (m, 1H), 2.26 (s, 3H), 1.65 (m, 3H), 0.99 (d, 3H, *J* = 6.9 Hz), 0.95 (d, 3H, *J* = 6.6 Hz). MS *m*/*z* 877.6 (M + H).

LY6Me-GM: ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.12 (s, 1H), 7.96 (d, 1H, *J* = 8.7 Hz), 7.25 (s, 1H), 6.94 (d, 1H, *J* = 12 Hz), 6.88 (d, 1H, *J* = 8.7 Hz), 6.57 (t, 1H, *J* = 11.1 Hz), 6.26 (bt, 1H), 5.88 (m, 2H), 5.42 (s, 1H), 5.17 (s, 1H), 4.72 (bs, 2H), 4.28 (d, 1H, *J* = 10 Hz), 4.07 (m, 2H), 3.83 (m, 4H), 3.55 (m, 7H), 3.32 (s, 3H), 3.25 (s, 3H), 2.69 (m, 2H), 2.39 (m, 1H), 2.24 (s, 3H), 2.01 (s, 3H), 1.84 (m, 2H), 1.79 (s, 3H), 1.74 (m, 4H), 1.57 (m, 4H), 0.99 (d, 3H, *J* = 6.9 Hz), 0.95 (d, 3H, *J* = 6.6 Hz). MS *m*/*z* 889.6 (M + H).

LY7Me-GM: ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.16 (s, 1H), 7.95 (d, 1H, *J* = 8.5 Hz), 7.26 (s, 1H), 6.94 (d, 1H, *J* = 12 Hz), 6.86 (d, 1H, *J* = 8.5 Hz), 6.57 (t, 1H, *J* = 11.5 Hz), 6.24 (bt, 1H), 5.87 (m, 2H), 5.43 (s, 1H), 5.19 (s, 1H), 4.85 (bs, 2H), 4.30 (d, 1H, *J* = 9.9 Hz), 4.15 (m, 2H), 3.83 (m, 4H), 3.55 (m, 2H), 3.46 (m, 5H), 3.35 (s, 3H), 3.26 (s, 3H), 2.62 (m, 2H), 2.35 (m, 1H), 2.25 (s, 3H), 2.00 (s, 3H), 1.82 (m, 2H), 1.78 (s, 3H), 1.72 (m, 3H), 1.44 (m, 6H), 0.99 (d, 3H, *J* = 6.9 Hz), 0.94 (d, 3H, *J* = 6.6 Hz). MS *m*/*z* 902.6 (M + H).

PI3K-1-GM: ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.12 (s, 1H), 8.08 (d, 1H, *J* = 7.9 Hz), 7.62 (d, 1H, *J* = 7.5 Hz), 7.27 (m, 2H), 6.93 (d, 1H, *J* = 11.3 Hz), 6.58 (t, 1H, *J* = 11.5 Hz), 6.37 (bt, 1H), 5.87 (m, 2H), 5.50 (s, 1H), 5.19 (s, 1H), 4.79 (bs, 2H), 4.29 (d, 1H, *J* = 9.8 Hz), 4.15 (bs, 1H), 3.85 (m, 4H), 3.72 (m, 2H), 3.54 (m, 5H), 3.33 (m, 1H), 3.27 (s, 3H), 3.26 (s, 3H), 2.66 (m, 4H), 2.41 (m, 1H), 2.08 (s, 3H), 2.00 (m, 1H), 1.78 (m, 3H), 1.67 (m, 5H), 1.01 (d, 3H, *J* = 6.9 Hz), 0.92 (d, 3H, *J* = 6.6 Hz). MS *m*/*z* 841.7 (M + H).

PI3K-2-GM: ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.09 (s, 1H), 8.14 (d, 1H, *J* = 7.9 Hz), 7.63 (d, 1H, *J* = 7.5 Hz), 7.33 (s, 1H), 7.31 (dd, 1H, *J* = 7.9 Hz, *J* = 7.5 Hz), 6.95 (d, 1H, *J* = 11.5 Hz), 6.58 (t, 1H, *J* = 11.7 Hz), 6.37 (bt, 1H), 5.88 (m, 2H), 5.50 (s, 1H), 5.21 (s, 1H), 4.74 (bs, 2H), 4.58 (m, 2H), 4.32 (d, 1H, *J* = 9.5 Hz), 3.91 (m, 1H), 3.82 (m, 4H), 3.55 (m, 6H), 3.36 (s, 3H), 3.28 (s, 3H), 2.72 (m, 2H), 2.41 (m, 1H), 2.04 (s, 3H), 1.80 (s, 3H), 1.72 (m, 1H), 1.03 (m, 6H). MS *m*/*z* 813.7 (M + H).

8. MCF-7 cells were serum starved for 24 h and stimulated with 1 μM insulin. Cell extracts were made in PI3K lysis buffer (137 mM NaCl, 20 mM Tris·HCl, pH 5, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10% v/v glycerol, 1% v/v Triton X-100) and the enzyme was immunoprecipitated using anti-p85 antibody (Upstate Biotechnologies Inc. no. 06-195). The complex was immobilized on Protein A beads (Amersham) and after several washes (3×1% Triton X-100 in PBS, 2×0.1 M Tris·HCl, pH 7.5, 0.5 M LiCl, 1×10 mM Tris·HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA) the immunoprecipitates were subjected to PI3-kinase activity assays. The lipid mix in 10 mM Hepes pH 7, 1 mM EGTA (final concentration 0.5 μM/mL) was incubated with the immobilized enzyme with or without drugs at room temperature for 15 min. The reaction was started upon addition of 10 μL of [γ-^{32}P]ATP (5 μCi/μL) and was allowed to proceed for 20 min at 37 °C after which was quenched by addition of 80 μL 1 M HCl. Lipids were extracted with 160 μL $CHCl_3/CH_3OH$ (1:1). The organic layer was applied to a pre-activated silica gel plate (EM Bioscience) and eluted with *n*-propanol/methanol/2 M AcOH (50:15:35). Plates were visualized by autoradiography and the lipids quantified using BioRad Gel Doc 1000 software.

9. SignaTECT® DNA-PK assay system from Promega was used with small modifications from the manufacturer's instructions. To each drug in 2 μL DMSO or to 2 μL DMSO were added 23 μL reaction mix [250 ng DNA from calf thymus (Sigma#D-3664), 0.2 mM biotinilated peptide substrate, 20 units of DNA-PK (Promega no. V5811), 1 μCi [γ-^{32}P]ATP,

2 μg BSA in 250 mM HEPES, pH 7.5, 500 mM NaCl, 50 mM $MgCl_2$, 1 mM EGTA, 0.5 mM EDTA and 5 mM DTT and the reaction mix was incubated for 10 min at 30 °C. After addition of 12.5 μL guanidine hydrochloride 7.5 M, each reaction was spotted to a SAM$^{2®}$ Biotin capture membrane (Promega no. 2861). After several washes with 2 M NaCl and 1% H_3PO_4 in 2 M NaCl, the samples were quantifies using a scintillation counter. For the interaction modulation assays the drugs were pre-incubated for 17 min on ice with 200 ng hsp90 α in PBS prior to the addition of the kinase mix.

10. GM was immobilized on Affigel 10 resin (BioRad) as described in ref 5. The GM-beads were blocked for 1 h at 4 °C with 0.5% BSA in TEN buffer (50 mM Tris·HCl pH 7.4, 1 mM EDTA, 1% NP-40) prior to use. Hsp90 α protein (Stressgen) was incubated with or without drugs for 20 min on ice. To each sample were added 20 μL GM-beads and the mixtures were rotated at 4 °C for 1 h followed by two washes with 500 μL ice cold TEN each. The GM-beads bound protein was eluted from the solid phase by heating in 35 μL 1 × SDS, analyzed by SDS/PAGE and visualized by immunoblotting with Hsp90 α (Stressgen no. SPA-840).

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LETTERS

Synthesis and Evaluation of Geldanamycin–Testosterone Hybrids

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Abstract—Geldanamycin (GDM) binds to the Hsp90 chaperone protein resulting in the degradation of several important signaling proteins. A series of GDM–testosterone linked hybrids has been synthesized and evaluated for activity against prostate cancer cell lines. The hybrid with the greatest activity exhibits potent and selective cytotoxicity against prostate cancer cells containing the androgen receptor. © 2000 Elsevier Science Ltd. All rights reserved.

Introduction

Geldanamycin[1] (GDM), first isolated from *Streptomyces hygroscopicus*, was originally identified as a potent inhibitor of cells transformed by the v-src oncogene.[2] It was later shown that GDM did not directly inhibit the src-encoded tyrosine kinase, but rather caused the proteosome dependent degradation of c-src and certain other signaling proteins.[2,3] GDM exerts its biological effects by binding to the highly conserved N-terminal ATP binding pocket of the molecular chaperone Hsp90.[4–7]

Hsp90[8] is an abundant chaperone protein that plays a role in the process of protein refolding and in the conformational maturation of several signaling molecules,[9] including steroid receptors,[10] Raf protein kinase,[11] and several transmembrane tyrosine kinases.[12,13] Occupancy of the Hsp90 pocket by GDM leads to the proteasomal degradation of these proteins and resultant cell death. GDM causes growth arrest and subsequent apoptosis of cancer cells and a related analogue is currently in early clinical trial.[14] However, the broad spectrum of activity of GDM against multiple important regulatory proteins suggests that its medicinal use will be limited by significant toxicity. As a result, we have initiated a program aimed at synthesizing and identifying derivatives of GDM that selectively inhibit or degrade particular proteins that are necessary for cancer growth.

We have previously shown that an appropriately fashioned hybrid that links estradiol (E2) and GDM (Fig. 1, below) demonstrated selective degradation of the estrogen receptor (ER) and HER2 kinase in MCF7 breast cancer cells, while having diminished effects against Raf-1 and IGF1R.[15] Our goal was to synthesize a GDM derivative, with less toxicity than GDM, which would be selectively cytotoxic toward breast cancer cells that express ER.

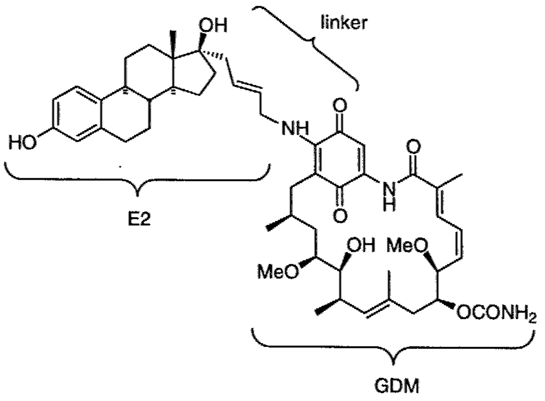

Figure 1. GDM-estradiol (E2) heterodimer that displays selective degradation of target cells containing ER.

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Since the AR is required for the unregulated growth of prostate carcinomas and tumor progression to an advanced, hormone-independent state is often accompanied by AR mutation, a compound that induces the selective degradation of the AR could be a useful therapeutic agent. In this report, we describe the synthesis of GDM–testosterone hybrids designed to selectively inhibit the androgen receptor.

Chemistry

The general strategy for linking GDM and testosterone is shown in Figure 2. Although limited SAR data is available for testosterone compared to E2, the C17-β-hydroxy group is still required for strong binding affinity.[16] Our strategy centered on the α-stereoselective addition of a lithiated alkynol to the C17-ketone of testosterone.[17] The hydroxyl could then be elaborated to a terminal primary amino group for coupling to GDM. Our laboratory[12] and others[18] have reported that the C17 methoxy of the benzoquinone undergoes smooth Michael-like reaction with amines. Previous SAR work[15] and a recent GDM-Hsp90 crystal structure[4] show that C17 of GDM is tolerant to a variety of substitution patterns.

In the event, testosterone was protected as its C3 cyclic ketal derivative. The ketalization step was accompanied by olefin migration to C5 (Scheme 1). Subsequent TPAP oxidation at C17 afforded ketone **2**. The addition of excess lithiated *t*-butyldimethylsilylalkynyl ether to the C17-ketone gave quantitative yields of the desired products, **3a–e**,[19] with exclusive addition from the α-face. Treatment of this product with TBAF followed by a three-step sequence afforded good yields of the azide, **5a–e**. Removal of the ketal with 1 N HCl in MeOH, also resulted in re-conjugation of the olefin. Finally, the azide was reduced with PEt$_3$ to afford the amines, **6a–e**, which were used immediately in the coupling with GDM. The hybrids **7a–e** were obtained in 75–100% yields.

The alkyne of the carbon linker was also modified as shown in Scheme 2, below. Conversion to the *E*-olefin was accomplished using LAH in THF to afford **8a**, while Lindlar reduction produced *Z*-olefin **8b**. Full saturation to the alkane without reducing the ring olefin was accomplished with Wilkinson's catalyst as demonstrated for substrate **8c**. The alcohols were then converted to desired hybrids **9a–c** as described in Scheme 1.

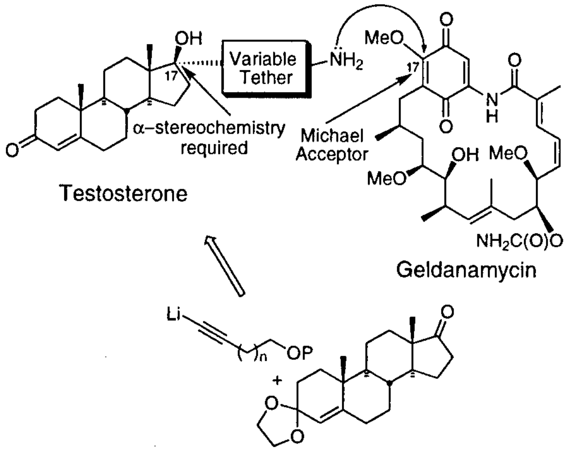
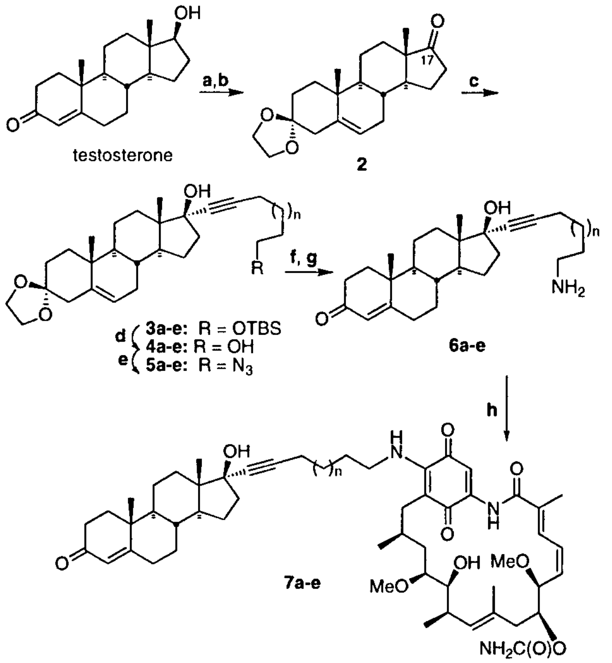


Figure 2. Structures and strategy for hybridization of testosterone and geldanamycin.

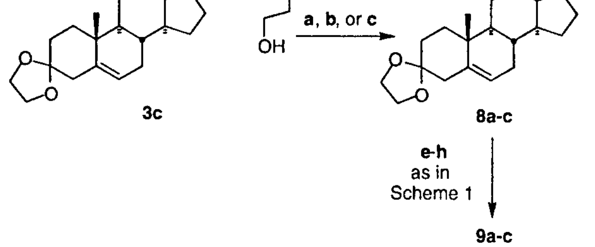
Biological Results

As part of our initial study hybrids **7a–e**, **9a–c**, and GDM were first evaluated for cytotoxicity against LNCaP prostate cancer cells as shown in Table 1.

A very clear SAR pattern based upon the size and nature of the linker can be discerned. The 6-carbon alkynyl hybrid, **7c**, is the most potent with an IC$_{50}$ of 100 nM compared to the parent GDM at 40 nM. The 5-carbon alkynyl linker is the next potent at 200 nM, however,

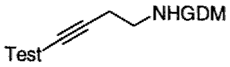
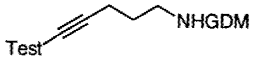
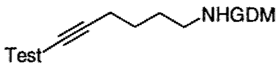
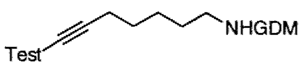
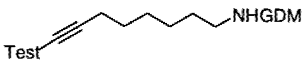
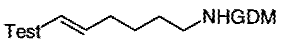
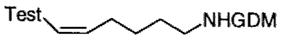
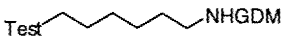


Scheme 1. (a) *p*-TSA, (CH$_2$OH)$_2$, heat, 80%; (b) TPAP, NMO, CH$_2$Cl$_2$, quantity; (c) TBS-alkynol, *n*-BuLi, pentane-THF, 0 °C, quantity; (d) TBAF, THF, 0 °C, quant.; (e) i. MsCl, TEA, CH$_2$Cl$_2$, ii. NaN$_3$, DMF; (f) 1N HCl, MeOH; (g) PEt$_3$, THF, H$_2$O; (h) GDM, DMSO, 2 h, 75–100% yield.



Scheme 2. (a) LAH, THF, reflux; (b) Pd/CaCO$_3$, EtOH, H$_2$, quinoline; (c) RhCl(PPh$_3$)$_3$, EtOAc, H$_2$.

Table 1. Cytotoxicity for GDM and new hybrids **7a–e** and **9a–c** in LNCaP prostate cancer cells

Compound	Linker	IC_{50}
GDM	--------	40 nM
7a		>1 μM
7b		200 nM
7c		100 nM
7d		>1 μM
7e		>1 μM
9a		>1 μM
9b		>1 μM
9c		>1 μM

shorter or longer tether lengths are less potent, all greater than 1 μM in potency. Further analysis of the alkyne functionality in the 6-carbon linker shows that it is absolutely crucial for potency. Reduction to the *E*-(**9a**) and *Z*-(**9b**) olefins, (or to the fully saturated system, **9c**) all result in complete abrogation of activity. We have previously observed the importance of the linker element on activity in the E2-GDM series as well, although the attachment in that series was at the C-16 position of E2.[15]

Table 2 compares the IC_{50}'s of GDM and **7c** on three prostate (two AR-dependent and one AR-independent), two breast (one ER-dependent and one ER-independent), and one colon (ER- and AR-independent) cancer cell line. In this experiment, both GDM and **7c** were potent against the LNCaP (mutated AR) and LAPC4 (wild-type AR) cell lines. However, most striking is the activity against the AR-independent PC3 cells, in which GDM was still very potent, but the hybrid structure **7c** *was 13 times weaker*. A similar trend was observed with MCF7 breast cancer cells (AR-independent), wherein **7c** was very weak ($IC_{50} = 300$ nM) while GDM was a potent inhibitor ($IC_{50} = 30$ nM).

Table 2. Enhanced cytotoxicity of the GDM and **7c** towards AR-dependent cells

Cell line	Origin	GDM IC_{50} (nM)	**7c** IC_{50} (nM)
LNCaP	Prostate	40	100
LAPC4	Prostate	25	40
PC3	Prostate	20	300
MCF7	Breast	30	300
MDA468	Breast	20	240
Colo205	Colon	20	200

Two important deductions can be drawn from the IC_{50} data. First, attachment of testosterone to GDM affords a compound that exhibits selected cytotoxicity for cells which are AR-dependent, demonstrating the success in our strategy of selectively targeting AR-dependent cells. Second, in AR-dependent cells, there is only a slight attenuation of activity of the hybrid compound **7c** compared to GDM. This is a particularly exciting result when one takes into account the attachment of the considerably large testosterone structure to the GDM nucleus.

The mechanism whereby the hybrid molecule, **7c**, selectively inhibits the growth of cancer cells that are AR dependent is currently under investigation. Preliminary data (not shown) suggests that **7c** is much less active than GDM in inducing degradation of the AR and other cellular proteins, but that it may inhibit AR function by causing the cytoplasmic sequestration (data not shown) of AR. These findings and the demonstrated selective inhibition of cell growth, suggest that **7c** may be less toxic than GDM while still retaining activity against prostate cancer. Furthermore, both GDM and **7c** induce the degradation of wild-type (LAPC4) and mutated (LNCaP) androgen receptor.[20] This finding suggests that the hybrid (**7c**) might be useful in the considerable portion of advanced stage, hormone-independent prostate cancers that express the mutated, activated form of AR.

Conclusion

We have designed and prepared a series of C17-C17 testosterone–GDM linked hybrids. While the potency of GDM is independent of the presence or absence of AR, one of our newly synthesized hybrid molecules, **7c**, exhibits strong and selective cytotoxicity towards prostate cancer cells expressing AR. Our current research efforts are centered toward determination of the mechanism of selective inhibition of AR by the GDM hybrid, **7c**.

We are also investigating the efficacy of the hybrid compound, **7c**, in an animal model to determine if the selectivity can be realized in vivo.

Acknowledgements

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Pamela Munster and Mr. Ezra Rosen for invaluable support and experimental assistance.

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20. Zheng, F. F. unreported results.

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LETTERS

SYNTHESIS AND EVALUATION OF GELDANAMYCIN-ESTRADIOL HYBRIDS

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Abstract: Geldanamycin (GDM) binds to the Hsp90 chaperone protein and causes the degradation of several important signalling proteins. A series of novel estradiol-geldanamycin hybrids has been synthesized and evaluated for their ability to induce the selective degradation of the estrogen receptor (ER). The hybrid compounds are active and more selective than the parent causing degradation of ER and HER2, but not other GDM targets.

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Introduction

The ansamycin antibiotic Geldanamycin[1] (GDM), first isolated from *Streptomyces hygroscopicus*, was originally identified as a potent inhibitor of src kinase.[2] Later work showed that GDM was not directly an inhibitor of this kinase, but that it inhibited kinase activity by inducing the proteosome dependent degradation of Src and other tyrosine kinases.[2,3] The ansamycins exert their biological effects by binding to a highly conserved pocket in the molecular chaperone Hsp90.[4-6] The latter is an abundant chaperone protein, required for protein refolding after stress and for the conformational maturation of some signalling molecules. Ansamycins inhibit these processes and induce the degradation of Hsp90 substrates.[7-9] The range of signalling proteins affected by GDM is expected to result in non-selective toxicity thereby compromising its applicability as a therapeutic agent. We have begun a program to prepare derivatives of GDM that would selectively induce the degradation of particular proteins.

In this connection, it was hoped that an appropriately fashioned hybrid drug linking estradiol (E2) and GDM would show higher affinity for estrogen receptor (ER). Our initial goal here was to first design and synthesize hybrid compounds and then check for activity in comparison to the parent GDM. The ultimate goal was to realize selectivity in the degradation of specific proteins. Particular targets might be the ER and HER2. HER2 is a transmembrane kinase that is amplified and overexpressed in a significant number of breast cancers. Successful targeting of these proteins could lead to arrested cell growth and/or apoptosis. We describe herein our preliminary results in synthesizing these compounds and resultant biological data.

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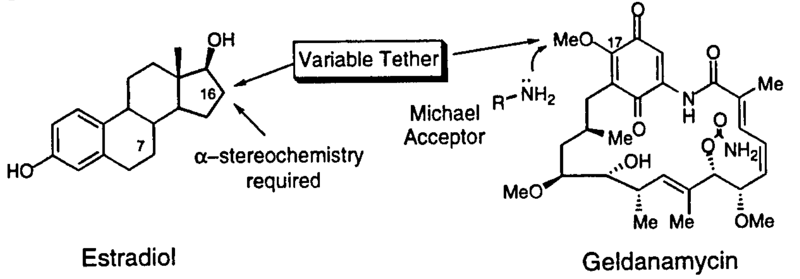
Our initial goal was to investigate the linkage on E2 and GDM in a manner that would allow for retention of activity for both components. Concerning the chemical linkage of the compounds, the site we investigated on estradiol[10] was the C-16[11] position. For high ER binding affinity the stereochemistry at C16 should be α.[12] As regard to GDM, our laboratory[9] and others[13] had reported that the C-17 methoxy of the benzoquinone undergoes smooth Michael-like reaction with amines. SAR data[13] has also shown C-17 to be tolerant to substitution and this was confirmed by a GDM-Hsp90 crystal structure.[4] Therefore, we required a strategy in which a linker element had to be constructed in an α-stereoselective fashion at C-16 of estradiol, with the provision that it could further be elaborated to a terminal primary amino group for coupling to GDM.

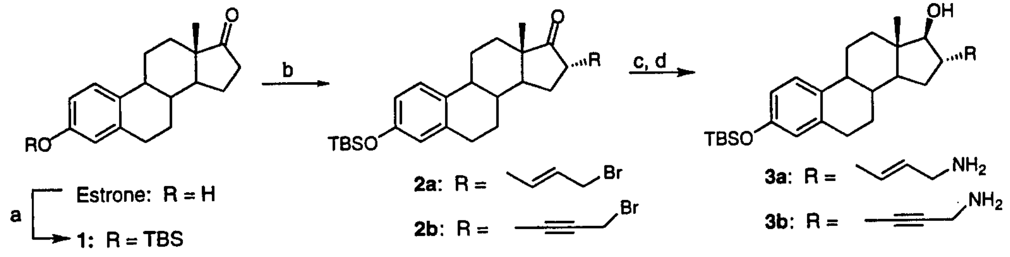
Figure 1. Structures and strategy for hybridization of estradiol and geldanamycin.



Chemistry

For connection at the C-16 position, a potentially problematic[14] α-alkylation of the C-17 ketone of estrone would be required. Our initial attempts to achieve this result via the C17 ketone enolate of TBS protected estrone **1** and unactivated electrophiles (alkyl bromides/iodides) resulted in poor yields, bis-alkylation, and predominantly β-products. However, Katzenellenbogen's method[15] using (*E*)-1,4-di-bromo-2-butene, 1,4-di-bromo-2-butyne, allylbromide, and carefully controlled temperatures, gave exclusively the desired α-alkylation products **2a-c** in fair to good yields. Following conversion of the resulting bromides to the azides by sodium azide, stereoselective reduction of the C-17 ketone at -78 °C followed by warming to -10 °C to reduce the azide, produced amino alcohols **3a** and **3b** in excellent yields.

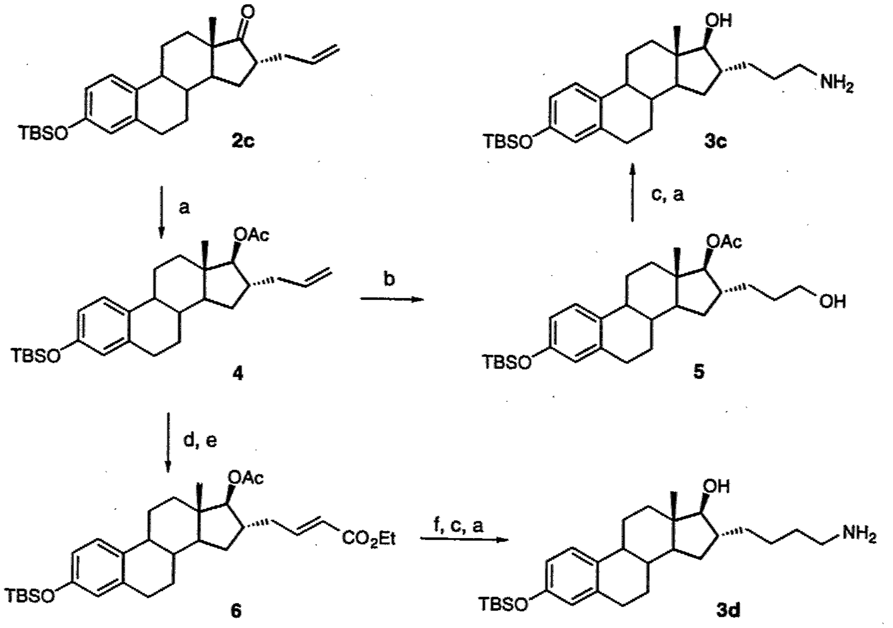
Scheme 1



Reagents: (a) TBSCl, imidazole, DMF, 86%; (b) i) LDA, THF, 0 °C, ii) electrophile, -35 °C, 12 h, 40-75%; (c) NaN_3, DMSO, THF, H_2O, 80-100%; (d) LAH, THF, -78 °C to -10 °C, 3 h, 100%.;

Again since alkyl halides were poor electrophiles, the allylated precursor **2c** was used as a handle for elaboration to prepare 3- and 4- carbon saturated linkers with the desired C-16α configuration (Scheme 2). Following selective LAH reduction to the C-17β alcohol, protection as the acetate afforded **4**. Hydroboration/sodium perborate oxidation of **4** afforded 3-carbon alcohol **5**. Conversion of **5** to the azide via the mesylate and subsequent LAH reduction of the azide and C-17 acetate provided amino alcohol **3c**. For the 4-carbon saturated linker, oxidative cleavage of **2c** with $OsO_4/NaIO_4$ followed by Horner-Emmons homologation and hydrogenation of the resulting olefin afforded **6** in 95% yield. Reduction of the esters and subsequent conversion of the primary alcohol to the amine afforded the fully saturated 4-carbon linker **3d**.

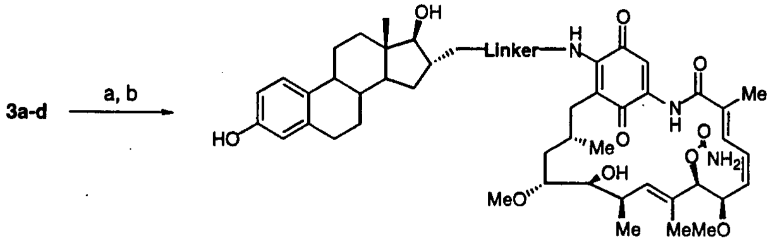
Scheme 2



Reagents: (a) i. LAH, THF, -78 °C to -10 °C, 3 hrs, quant. ii. Ac_2O, DMAP, CH_2Cl_2, 95%; (b) i. 9-BBN, THF, ii. $NaBO_3$, H_2O, 70%; (c) i. MsCl, Pyr, ii. NaN_3, DMF, 80%; (d) i. OsO_4, NMO, ii. $NaIO_4$, THF; (e) $Ph_3P{=}CHCO_2Et$, PhH; (f) i. Pd/C, H_2, EtOAc, 3 steps-95%, ii. LAH, THF, 0 °C

For coupling to GDM, we relied on the aforementioned Michael behavior at the C-17 position with primary amines.[16] In the coupling event, amino-alcohols **3a-d** underwent smooth reaction with GDM in DMSO overnight, followed by deprotection of the phenolic TBS ether with TBAF-HOAc to afford estradiol-GMD hydrids **7a-d** cleanly in fair to good yields (40-60%). The coupling proves very clean and unreacted GDM can be recovered from the reaction mixture. The use of an excess (2.0 equiv.) of amino alcohols **3** gave much higher yields of **7a-d** (>85%).

Scheme 3



7a-d

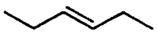
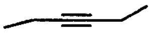
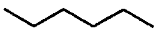
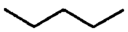
Reagents: (a) Geldanamycin, DMSO; (b) TBAF-HOAc, THF.

Biological Evaluation of Constructs

For initial evaluation, the effects of GDM and **7a-d** on the steady-state levels of HER2, ER, and Raf-1 in MCF7 breast cancer cells were measured. The activity of was quantitated by determining the concentration of each compound needed to reduce the protein expression by 50% (i.e. the IC_{50}-HER2).[17] Results are shown in Table 1.

GDM was, as expected, the most active against all three proteins, particularly HER2 and ER. It is also clear that the hybrid drugs do indeed retain activity. The extent of this proves to be very strongly dependent on the nature of the tether or linker between the estradiol and GDM. For example, analog **7a** with the *E*-2-butene linkage, is the most active of the series. Going from an alkene to an alkyne in the linker for **7b** results in almost identical activity (except against ER where a slight decrease was noted). Most dramatic is the effect of saturation of the linker or shortening to 3-carbons in analogs **7c** and **7d**. For these two compounds, all activity is essentially lost. Thus, the activity of the constructs is surprisingly sensitive to the nature of the unsaturation in the linker element.

Table 1. The effects of GDM and **7a-7d** on steady state levels of HER2, ER, and Raf-1 in MCF7.

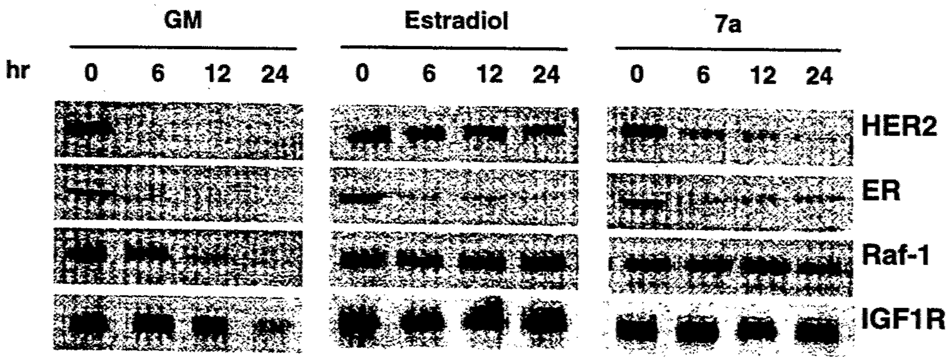
Compound	Linker	IC_{50}-HER2 (μM)	IC_{50}-ER (μM)	IC_{50}-Raf (μM)
GDM	-	0.05	0.06	0.2
7a		0.1	0.08	1.5
7b		0.1	0.22	1.5
7c		>2000	>2000	>2000
7d		>2000	>2000	>2000

Since these new hybrids do exhibit activity, a comparison of the effects of GDM, estradiol, and construct **7a** on HER2, ER, Raf-1, and IGF1R was made in MCF7 cells. The results are shown in Figure 2.[18]

GDM causes rapid degradation of HER2 and ER after 6 hr, and Raf-1 and IGF1R after a slightly longer time period. E2 is known to induce the downregulation of its own receptor.[19] E2 and **7a** both cause downregulation of ER in MCF7. As expected, treatment of MCF7 cells with E2 affected the steady state levels of ER, while having no effect on the other proteins. The effects of E2 on ER are complex and involve an interaction with the ER promoter. However, E2 stimulates breast cancer cell growth and **7a** selectively inhibits the growth of ER-containing cells.[20] The data suggest that induction of degradation of ER by **7a** is not preceded by receptor activation. Lead compound **7a** however, still exhibits very good activity against HER2 and ER, albeit somewhat attenuated compared to GDM.

Most remarkable is the fact that **7a** has essentially no effect on IGF1R and reduced activity against Raf-1[21] indicating that hybridization of GDM and estradiol does indeed exhibit selectivity. That is **7a** appears to be targeting the ER-Hsp90 interaction exclusively leaving other Hsp90 associated proteins unaffected. This could lead to an improved therapeutic profile compared to GDM. For example, Raf-1 represents an important intermediate of several transduction pathways. GDM analogs, with reduced activity against Raf-1, are desirable as they may prove to be less toxic to normal cells. In addition, in a prostate cancer cell line, GDM induced the loss of androgen receptor; **7a** had no effect confirming its specificity.[20]

Figure 2. The effects of GDM, E2, and **7a** on the steady state levels of HER2, ER, Raf-1, and IGF1R.



We note that these same linkers have previously been reported for attaching fluorescent labels/radiotracers to the C-16α position as well as to other estradiol derivatives.[22] The binding affinity of these constructs was also strongly dependent upon the nature of the tether, with the four carbon unsaturated ligands (corresponding to **7a** and **7b**) showing the highest degree of binding for ER. It is therefore likely that the activity of our compounds is a consequence of binding to ER otherwise the role of the linker should not play such an important effect. Further experiments to investigate these hypotheses, *in vivo* models, and optimization of hybrid structure are actively underway.

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16. We have observed that primary amines give a faster reaction that secondary ones, with the exception being secondary amines constrained in a ring (3-6 membered).
17. MCF-7 cells were treated with various concentrations of GDM or **7a** - **d** for 24 h. The same amounts of cell lysates were separated by electrophoresis. Steady state levels of HER2, ER, and Raf-1 were measure by immuno-blotting with specific antibodies, quantitated using Gel Doc 1000 (Bio-Rad). IC_{50} is the amount of each protein to inhibit protein expression by 50% compared to the control.
18. MCF-7 cells were treated with different compounds (1 mM) for different time periods as indicated. Total cell lysates were blotted with specific antibodies.
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Advances in Brief

Identification of a Geldanamycin Dimer That Induces the Selective Degradation of HER-Family Tyrosine Kinases[1]

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Abstract

Geldanamycin (GM) is a natural antibiotic that binds Hsp90 and induces the degradation of receptor tyrosine kinases, steroid receptors, and Raf. It is a potent inhibitor of cancer cells that overexpress HER-kinases, but its effects on other important proteins may cause significant toxicity and limit its clinical use. We report the synthesis and identification of a GM dimer, GMD-4c, which had selective activity against HER-kinases. Selectivity was a function of linker length and required two intact GM moieties. GMD-4c is a potent inducer of G_1 block and apoptosis of breast cancer cell lines that overexpress HER2, but does not appreciably inhibit the growth of 32D cells that lack HER-kinases. GMD-4c could be useful in the treatment of carcinomas dependent on HER-kinases.

Introduction

HER-family transmembrane receptor tyrosine kinases play an important role in transducing extracellular growth signals and when activated can be oncogenic (1, 2). Overexpression of HER1 and HER2 occurs in a variety of human malignancies. In breast cancer, overexpression of HER2 is associated with a poor prognosis (2). HER1 and HER2 are attractive targets for therapeutic development. Antibodies against each of these receptors have been shown to have antitumor effects in animal models (3). Recently, an anti-HER2 antibody was shown to be effective in the treatment of breast cancers in which HER2 is overexpressed (2, 4). However, therapeutic effects were seen in only a minority of patients and were usually short-lived. Other, more effective methods for HER2 inhibition are needed.

GM[3] and herbimycin A are benzoquinoid ansamycin antibiotics (5). This class of drugs binds to a specific pocket in the chaperone protein Hsp90 (6, 7). Occupancy of this pocket by the drug leads to the degradation in the proteasome of a subset of proteins that require Hsp90 for conformational maturation (8–11). These include the HER- and insulin-receptor families of tyrosine kinases, Raf-1 serine kinase, and steroid receptors. The addition of GM to tumor cells leads to a Rb-dependent G_1 growth arrest and apoptosis.[4] HER-kinases are the most sensitive targets of GM, and tumor cell lines in which HER2 is overexpressed are inhibited by especially low concentrations of the drug (12–14). These findings imply that GM and related drugs may be useful in the treatment of a variety of tumors. An analogue of GM, 17-allylaminoGM, is currently under Phase I clinical trials. However, the number of important signaling molecules that are affected by ansamycins suggests that they may have untoward toxicity.

We have endeavored to synthesize derivatives of GM that have a narrower spectrum of action and greater selectivity. Signaling via the HER-kinases may require their association with Hsp90. This chaperone is required for *sevenless* (*Drosophila* epidermal growth factor receptor-family member) signaling (15). v-Src associates with Hsp90 and is very sensitive to GM (6). Both the sensitivity of v-Src to ansamycins and its association with Hsp90 depend on the presence of the catalytic domain but do not require catalytic activity (16, 17). The sensitivity of HER2 to GM also requires the catalytic domain (12). However, a direct interaction of Hsp90 and HER-kinases has not been convincingly demonstrated.

These data suggested to us that Hsp90 is likely to interact with the catalytic domain of HER-kinases. Because HER-kinases undergo dimerization on activation, we speculated that each element of the HER-kinase dimer interacts with Hsp90. Accordingly, it seemed possible that a GMD might be able to interact with both subunits of the HER-kinase dimers. Here, we report the synthesis and evaluation of several GMDs and the identification of a GMD, GMD-4c, which induces the selective degradation of HER-kinases.

Materials and Methods

Cell Lines. The human breast cancer cell lines MCF-7 and SKBR-3 were obtained from American Type Culture Collection (Manassas, VA) and maintained in DME/F12 (1:1) supplemented with 10% heat-inactivated FBS (Gemini Bioproducts), 2 mM glutamine, and 50 units/ml each of penicillin and streptomycin, in a humidified 5% CO_2/air atmosphere at 37°C. The murine hematopoietic cell line 32D was kindly provided by Dr. Yosef Yarden (The Weizmann Institute of Science, Rehovot, Israel) and maintained in RPMI 1640 supplemented with 10% heat-inactivated FBS, 2 ng/ml interleukin 3 (R&D Systems, Inc.), 2 mM glutamine, and 50 units/ml each of penicillin and streptomycin.

Antibodies. Polyclonal antibodies against HER2 (c-18), HER3 (C-17), Raf-1 (c-12), and PI3 kinase (p85) (Z-8) were purchased from Santa Cruz Biotechnology, Inc. The HER2 monoclonal antibody (Ab-5) for immunoprecipitation was from Oncogene. A monoclonal antibody against ER (clone H-151) was from *Stress*Gen Biotechnology Corp. A polyclonal antibody against the α-subunit of IGF-IR was kindly provided by Dr. L-H. Wang (Mt. Sinai Medical Center, New York, NY).

GM and Its Analogues. GM was kindly provided by Drs. David Newman and Edward Sausville (Drug Synthesis and Chemistry Branch, National Cancer Institute, Bethesda, MD), dissolved in 100% DMSO, and stored at −20°C. The GM analogues were prepared according to S. Kuduk *et al.*[5] using the method of Schnur *et al.* (13). Briefly, the GMDs were prepared by treatment of GM with 0.5 equivalent of the appropriate diamine in DMSO. The ansa-ring-opened GMDs (GMD-a and GMD-aa) were prepared by methanolysis (NaOMe/methanol) of the GMD-4c. GM-quinone was synthesized by first

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[3] The abbreviations used are: GM, geldanamycin; GMD, GM dimer; ER, estrogen receptor; IGF-I, insulin-like growth factor I; IGF-IR, IGF-I receptor; FBS, fetal bovine serum; RB, retinoblastoma protein.

[4] M. Srethapakdi and N. Rosen, manuscript in preparation.

[5] S. D. Kuduk *et al.*, unpublished data.

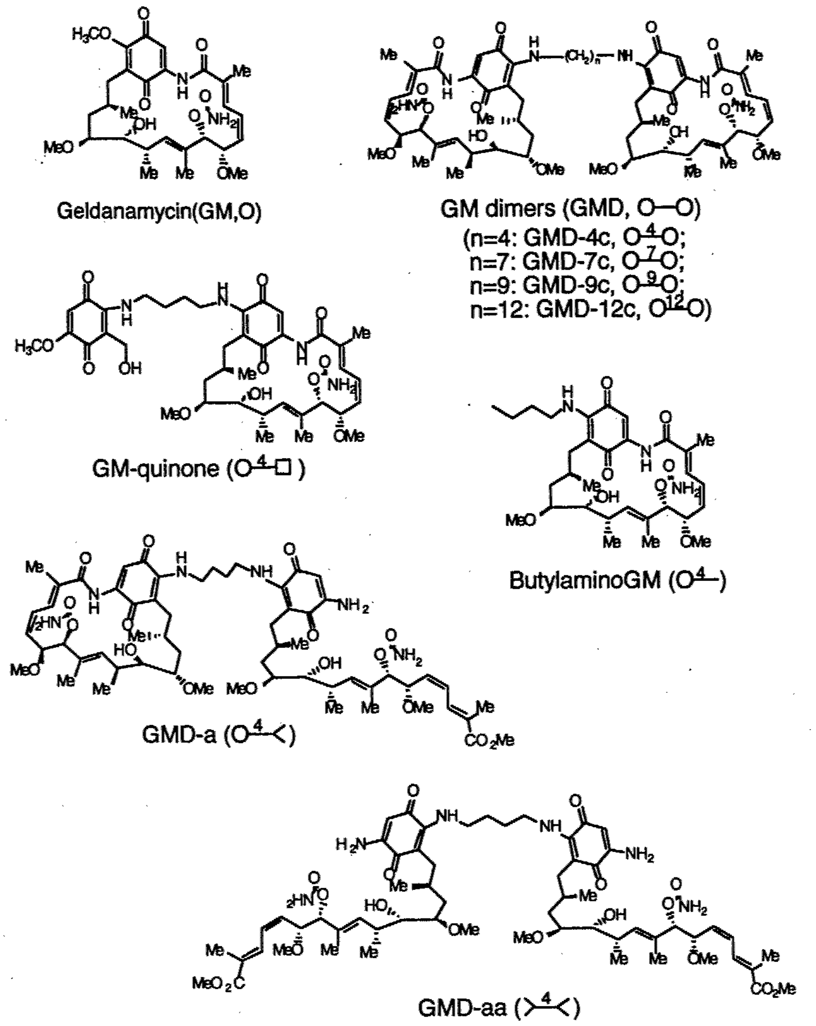
Geldanamycin(GM,O)
GM dimers (GMD, O—O)
(n=4: GMD-4c, O$\overset{4}{—}$O;
n=7: GMD-7c, O$\overset{7}{—}$O;
n=9: GMD-9c, O$\overset{9}{—}$O;
n=12: GMD-12c, O$\overset{12}{—}$O)
GM-quinone (O$\overset{4}{—}$□)
ButylaminoGM (O$\overset{4}{—}$)
GMD-a (O$\overset{4}{—}$<)
GMD-aa (>$\overset{4}{—}$<)

Fig. 1. Chemical structures of GM and its analogues, constructed according to "Materials and Methods." O, GM moiety; –, carbon linker; □, quinone; < or >, GM moiety with an opened ansa-ring.

treating GM with excess 1,4-diamobutane, then the addition of 2-methoxy-1-hydroxymethylquinone.

Cell Growth Experiments. Cells were plated in 6-well tissue-culture plates (Corning Glass) at 20,000 cells/well. Two days after plating, cells were treated with different concentrations of drugs or the vehicle DMSO (0.1%). MCF-7 and SKBR-3 cells were treated for 4 days. Medium with the appropriate drug or vehicle was changed every 2 days. Cells were trypsinized, collected, and counted on a Coulter counter. IC_{50} for cell growth is designated as the amount of each drug needed to inhibit cell growth by 50% compared with the control vehicle. 32D cells were treated and counted every day for 3 consecutive days using a hematocytometer.

Immunoprecipitation and Immunoblotting. MCF-7 cells were washed twice with ice-cold PBS, collected by scraping, and transferred into microcentrifuge tubes. For immunoblotting (HER2, HER3, Raf-1, ER), cells were lysed with SDS lysis buffer [50 mM Tris-HCl (pH 7.5), 2% SDS, 10% glycerol, and 1 mM DTT], boiled for 10 min, and sonicated briefly. For immunoprecipitation (HER2 and IGF-IR), cells were lysed with NP40 lysis buffer [50 mM Tris-HCl (pH 7.5), 1% NP40, 150 mM NaCl, 1 mM Na_3VO_4, 40 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of aprotinin, leupeptin, and soybean trypsin inhibitor] for 20 min at 4°C. Cell lysates were cleared by centrifugation at 14,000 × *g* for 15 min at 4°C in a microcentrifuge. Supernatants were collected as the experimental samples. Protein concentration in each sample was determined using the BCA kit (Pierce Chemical Co.), according to the manufacturer's instructions. For detecting IGF-IR, samples were immunoprecipitated with anti-IGF-IR antibody. Immunocomplexes were collected on protein A-Sepharose beads (Pharmacia) and washed three times with the lysis buffer. Samples were subjected to SDS-PAGE, electrotransferred to nitrocellulose membranes, detected using the ECL kit (Amersham Corp.), according to the manufacturer's protocol, and quantitated using the Gel Doc 1000 (Bio-Rad Laboratories). IC_{50} for protein degradation is designated as the amount of each drug needed to decrease 50% of the protein (HER2 or Raf-1) compared with the control in MCF-7 cells after a 24-h treatment.

Pulse-labeling and Pulse-chase Experiments. To study the effects of GM and GMD-4c on protein synthesis, MCF-7 cells were pulse-labeled with

Table 1 *The effects of GM analogues on protein down-regulation and cell growth*

	MCF-7			SKBR-3
Drug[a]	IC_{50}[b] (HER-2) (nM)	IC_{50}[b] (Raf-1) (nM)	IC_{50}[c] (growth inh.) (nM)	IC_{50}[c] (growth inh.) (nM)
GM (O)	45	200	25	3
GMD-4c (O$\overset{4}{—}$O)	60	2200	100	20
GMD-7c (O$\overset{7}{—}$O)	70	500	600	200
GMD-9c (O$\overset{9}{—}$O)	500	3800	700	500
GMD-12c (O$\overset{12}{—}$O)	750	3500	700	650
ButylaminoGM (O$\overset{4}{—}$)	80	350	600	350
GM-quinone (O$\overset{4}{—}$□)	55	350	350	60
GMD-a (O$\overset{4}{—}$<)	500	3500	650	250
GMD-aa (>$\overset{4}{—}$<)	>5000	>5000	>2000	>2000

[a] See Fig. 1 for the structure of each drug.

[b] MCF-7 cells were treated with various concentrations of each drug; IC_{50} for each protein is designated as the amount of each drug needed to decrease the steady-state level of either HER2 or Raf-1 to 50% of the control after 24 h of treatment.

[c] MCF-7 and SKBR-3 cells were treated with different concentrations of each drug for 4 days; IC_{50} is designated as the amount of each drug needed to inhibit cell growth by 50% compared with the control after the 4-day treatment. The numbers were the average of three different experiments.

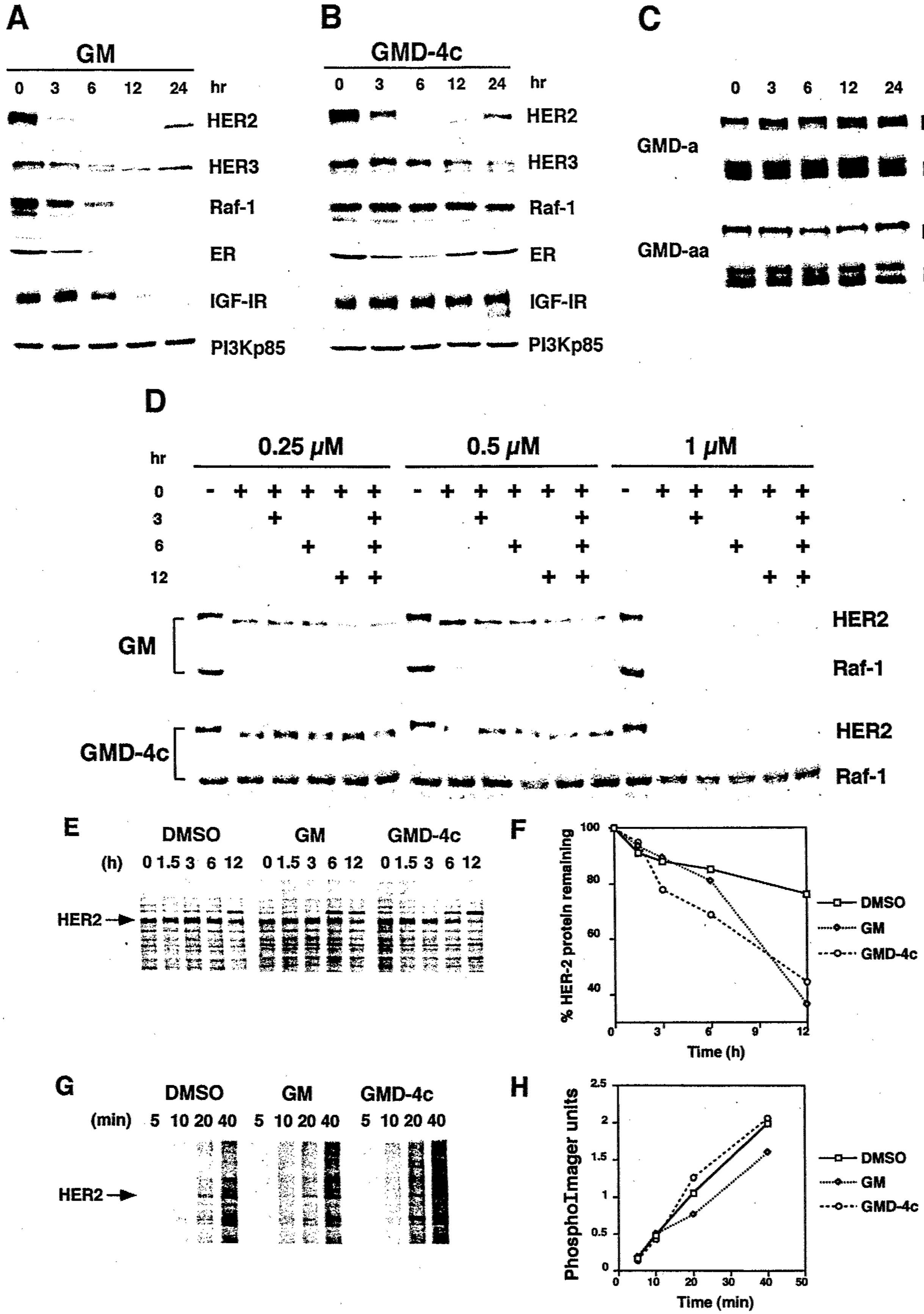

Fig. 2. The effects of GM and its analogues on the expression of the cellular proteins of MCF-7 cells. To investigate the effects of GM and its analogues on the steady-state levels of proteins, cells were treated with various drugs (each 1 μM) for different periods of time (*A*, *B*, and *C*), or cells were treated with either GM or GMD-4c, which was added multiple times according to the schedule shown on the *top* (*D*). + and hr, the time when the drug was added. Cells were treated for a total of 24 h. Total cell lysates were then extracted, subjected to SDS-PAGE, transferred to nitrocellulose membrane, and analyzed by immunoblotting using specific antibodies against each protein. To investigate the effect of GMD-4c on HER2

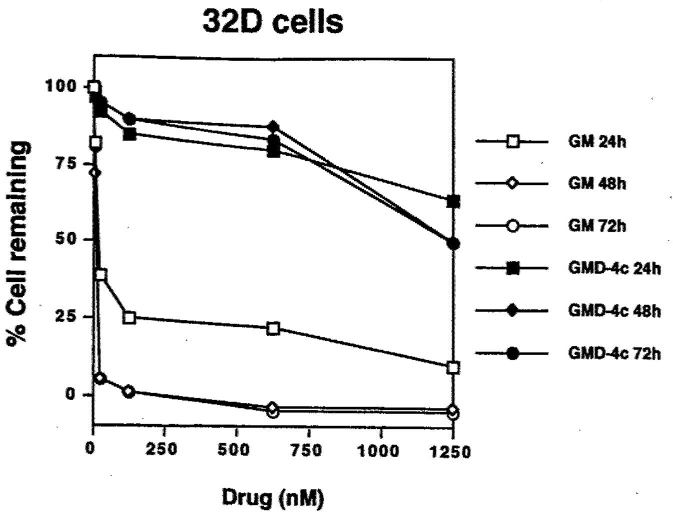

Fig. 3. The effects of GM and GMD-4c on the growth of the hematopoietic 32D cells, which lack HER-kinases. 32D cells were treated with various concentrations of either GM or GMD-4c for different periods of time. Cells were counted using a hemacytometer. The results are the average of three different experiments.

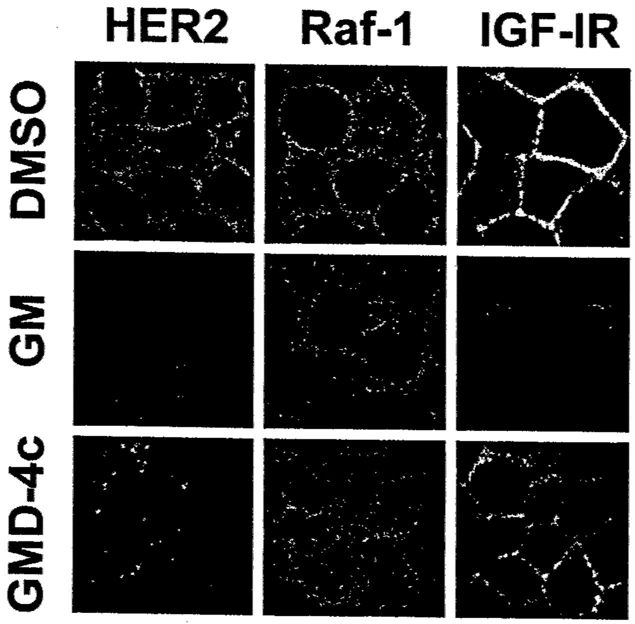

Fig. 4. Immunohistochemical analysis of the effects of GM and GMD-4c on the expression of HER2, Raf-1, and IGF-IR in MCF-7 cells. Cells were treated with either GM (1 μM), GMD-4c (1 μM), or the control vehicle DMSO (0.1%) for 24 h. Cells were then fixed and immunostained for HER2, Raf-1, and IGF-IR, according to "Materials and Methods."

[^{35}S]protein-labeling mix (NEN; 100 μCi/ml, 1175 Ci/mmol) in methionine/cysteine-free media for increasing amounts of time in the presence of either GM (1 μM), GMD-4c (1 μM), or the carrier (DMSO, 0.1%). To study the effects of GM and GMD-4c on protein degradation, MCF-7 cells were pulse-labeled to isotopic equilibrium with [^{35}S]protein-labeling mix (NEN; 100 μCi/ml, 1175 Ci/mmol) in methionine/cysteine-free medium mixed with regular medium/5% FBS (9:1) for 14 h and chased with unlabeled methionine/cysteine (150 μg/ml) in the presence of either GM (1 μM), GMD-4c (1 μM), or the carrier (DMSO, 0.1%) for a period of 12 h. Cells were collected at different time points from the pulse-labeling and pulse-chase experiments and lysed with NP40 lysis buffer. Samples containing equal amounts of protein (300 μg) were immunoprecipitated for HER2 and subjected to 7% SDS-PAGE. Gels were dried and exposed to X-ray films. HER2 bands were quantitated using Bio-Rad Gel Doc 1000.

Immunohistochemistry. Cells were grown and treated on fibronectin-coated coverslips placed in multiwell plates. Cells were fixed for 20 min at −20°C in 100% methanol, rehydrated in PBS for 10 min at room temperature, and blocked for 30 min at 37°C in a blocking solution consisting of 2% BSA, 10% normal goat serum, and 0.05% Tween 20 in PBS. HER2 and Raf-1 were immunodetected with antibodies (SC284 and SC133, respectively) from Santa Cruz Biotechnology, Inc., and IGF-IR was immunodetected with an antibody (Ab1) from Calbiochem Oncogene Science. Slides were incubated with a 1:1000 dilution of the primary antibody in blocking buffer for 1 h at room temperature, followed by an incubation with Alexa 546-coupled goat antirabbit IgG secondary antibody (A-1 I010; Molecular Probes) at a 1:50 dilution in blocking buffer, or fluoresceine isothiocyanate-coupled goat antimouse IgG (F276; Molecular Probes) at a 1:100 dilution. Slides were washed three times with 1 ml of 0.5% BSA in 0.05% Tween 20 in PBS in between and after incubations with primary and secondary antibodies. DNA was stained with bisbenzimide, which was included in the secondary antibody solution (3 μg/ml final concentration). Coverslips were mounted on glass slides using Vectashield (Vector Laboratories, Inc.) to prevent quenching of fluorescence. Immunofluorescence was detected with a Zeiss epifluorescence microscope at ×40 and ×100, using appropriate filters for detection of rhodamine and bisbenzimide, and via confocal microscopy.

Results and Discussion

We synthesized a series of GMDs covalently joined by alkylamino linkers of varying chain length (Fig. 1). The linker is bonded to the 17-carbon of both GM moieties. The crystal structure of GM bound to Hsp90 shows that the 17-carbon is the only one not buried in the binding pocket (7). The activities of GMDs were compared with those of GM and assessed in terms of efficiency of induction of down-regulation of the HER2 and Raf-1 protein kinases in MCF-7 cells (Table 1). The properties of these GMDs were found to vary as a function of chain length. GM itself causes the induction of HER2 degradation with an IC_{50} of 45 nM. Dimers with linkers of four to seven carbons retain activity against HER2 (IC_{50}, 60–70 nM). Dimers with longer linkers lose activity; the 12-carbon-linked compound has an IC_{50} of 750 nM.

The four-carbon-linked dimer, GMD-4c, has selective activity. GM causes Raf-1 degradation with an IC_{50} of 200 nM. GMD-4c is much less active, with an IC_{50} of 2200 nM. Selectivity is lost with increasing chain length; the seven-carbon-linked-dimer (GMD-7c) retains activity against HER2 (IC_{50}, 70 nM) and is only slightly less active than GM against Raf-1 (IC_{50}, 500 nM). As linker carbons increase to more than eight, activity against both targets declines in parallel. The properties of GMD-4c were examined in greater detail. GM causes the degradation over time of HER-kinases, Raf-1, the ER, and, more slowly, the IGF-IR (Fig. 2*A* and Fig. 4, and HER1 and HER4 data not shown). GMD-4c reduces HER2 expression with the same kinetics both on immunoblot and by immunohistochemical analysis (Fig. 2*B*). GMD-4c also decreases HER3 expression (Fig. 2*B*) and HER1 and HER4 expression (data not shown). GMD-4c is not selective for individual members of the HER-kinase family. However, under these conditions (1 μM, 24-h treatment), GMD-4c does not affect Raf-1 or IGF-IR expression. ER levels declined transiently but returned to

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protein degradation, cells were pulse-labeled with [^{35}S]protein-labeling mix (NEN; 100 μCi/ml, 1175 Ci/mmol) in methionine/cysteine-free medium mixed with regular medium/5% FBS (9:1) for 14 h and chased with unlabeled methionine/cysteine (150 μg/ml) in the presence of either GM (1 μM), GMD-4c (1 μM), or the carrier (DMSO, 0.1%) for a period of 12 h. Cell lysates from different time points were immunoprecipitated for HER2 protein, subjected to SDS-PAGE, followed by autoradiography (*E*), and quantitated by the Bio-Rad gel doc (*F*). To investigate the effect of GMD-4c on HER2 protein synthesis, cells were pulse-labeled with [^{35}S]protein labeling mix (NEN; 100 μCi/ml, 1175 Ci/mmol) in methionine/cysteine-free media for an increasing amount of time in the presence of either GM (1 μM), GMD-4c (1 μM), or the carrier (DMSO, 0.1%). Cell lysates from different time points were immunoprecipitated for HER2 protein, subjected to SDS-PAGE, followed by autoradiography (*G*), and quantitated by the Bio-Rad gel doc (*H*).

baseline by 24 h (Fig. 2*B*). Both GM and GMD-4c do not affect PI3-kinase (PI3Kp85) expression. A faster migrating HER2-immunoreactive band appeared after 12 h of treatment with either drug, but more prominently with GMD-4c (Fig. 2, *A* and *B*). This form accumulates in intracellular vesicles and corresponds to immature HER2. Glycosylation studies revealed that this HER2 form is partially glycosylated and sensitive to endoglycosidase H (data not shown; Ref. 18).

Additional GM derivatives were synthesized to explore the mechanism of selectivity (Fig. 1). ButylaminoGM, a molecule in which the four-carbon linker is attached to only one geldanamycin residue, and a four carbon-linked heterodimer of GM and a quinone (GM-quinone) are modestly weaker than GM against both HER2 and Raf-1; they are not selective (Table 1). GMD-aa, a GMD-4c derivative in which the ansa-ring of each of the GM moieties is opened, is inactive (Fig. 2*C*). GMD-a, a dimer in which the ring of only one of the GM moieties is open has much reduced activity against both targets (Table 1; IC_{50}, HER2 500 nM, and IC_{50}, Raf-1 3500 nM; Fig. 2*C*). These data suggest that the selectivity of GMD-4c depends on both GM moieties. This apparent selectivity could be a property of a weaker or more rapidly metabolized drug that might seem to have selective activity against the most sensitive target (HER2). To address this question, GM and GMD-4c were added to cells at different concentrations and frequencies (Fig. 2*D*). Even when GMD-4c was added at high concentrations four times in 12 h, it retained selectivity.

To investigate the mechanism by which GMD-4c down-regulates HER-family protein expression, we tested the effects of GM or GMD-4c on HER2 protein degradation and synthesis by pulse-chase and pulse-labeling experiments. Both GMD-4c and GM accelerated the degradation of HER2 protein (Fig. 2, *E* and *F*) and did not affect its rate of synthesis (Fig. 2, *G* and *H*). These results indicate that GMD-4c, like GM (9), affects HER2 expression by inducing protein degradation.

GMD-4c was a potent inhibitor of growth of breast cancer cells containing HER-kinases (Table 1), with an IC_{50} of 100 nM against MCF-7 cells compared with an IC_{50} of 25 nM for GM and 650 nM for GMD-a. SKBR-3, a cell line in which the *HER2* gene is amplified and the protein is highly overexpressed, is especially sensitive to both GMD-4c and GM (Table 1; IC_{50}, 20 nM and 3 nM, respectively). Most epithelial cancer cell lines express one or more members of the HER-kinase family. To assess whether the effects of GMD-4c on cells were specific, we used the 32D hematopoietic cell line (19). None of the members of the HER-kinase family are expressed in this murine interleukin-3-dependent myeloid progenitor cell line. GM is a potent inhibitor of 32D (IC_{50}, 3 nM), but GMD-4c does not appreciably affect its growth at concentrations up to 1 μM (Fig. 3).

Thus, GMD-4c induces the selective degradation of HER-family kinases and specifically inhibits the growth of HER-kinase containing tumor cell lines. Because its effects on other key signaling proteins are attenuated, GMD-4c is likely to be much less toxic than GM. This work supports the idea that selective ansamycins with a different, more restricted spectrum of targets than the parent molecules can be synthesized. In this case, the mechanism of selectivity is not yet known, but depends on the presence of both GM moieties and is a function of the linker length. GMD-4c may selectively interact with HER-kinase dimers, but it is also possible that it preferentially interacts with different Hsp90-family members than GM. This work represents a new strategy for abrogating growth receptor function in human tumors.

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